AD_______________

Award Number: DAMD17-01-1-0455

TITLE: DNA Vaccination to Overcome Peripheral Tolerance

PRINCIPAL INVESTIGATOR: Wei-Zen Wei, Ph.D.

CONTRACTING ORGANIZATION: Wayne State University
Detroit, Michigan 48202

REPORT DATE: August 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Annual (1 Aug 2002 - 31 Jul 2003)

4. TITLE AND SUBTITLE
DNA Vaccination to Overcome Peripheral Tolerance

5. FUNDING NUMBERS
DAMD17-01-1-0455

6. AUTHOR(S)
Wei-Zen Wei, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wayne State University
Detroit, Michigan 48202

E-Mail: weiw@karmanos.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)
The vaccination efficacy of DNA encoding atuologous rat ErbB-2 (neu) or heterologous human ErbB-2 (Her-2) is compared in rat neu transgenic mice (BALB neuT). Cross reactivity between Her-2 and rat neu was tested initially by immunizing normal mice, twice, i.m. with pEFBosGM-CSF and pCMVE2TM orpCDneuTM. E2TM and neuTM encodes the extracellular (ECD) and transmembrane (TM) domains of Her-2 and neu, respectively. Immunized mice were challenged with mammary tumor D2F2 expressing Her-2 (D2F2/E2) or neu (D2F2/neu). All mice immunized with E2TM or neuTM rejected tumors expressing the corresponding antigen. There is significant cross-protection against tumors expressing the non-corresponding ErbB-2, although the antibodies demonstrated little corss-reactivity. When tested in neuT mice which are tolerant to rat neu, neuTM but not E2TM delayed spontaneous tumorigenesis. Therefore, Her-2 and rat neu are cross-reactive antigens in normal mice, but only autologous neu, not heterologous Her-2 induced protective immunity in NeuT transgenic mice. This may indicate a lack of cross-reactivity of antibodies which are critical in inhibiting NeuT tumor. To enhance the immunogenicity of autologous ErbB-2, an adjuvant sequence of Pan DR Reactive Epitope (PADRE) has been cloned into human ErbB-2 and the immunogenicity is being tested.

14. SUBJECT TERMS
No Subject Terms Provided.

15. NUMBER OF PAGES
47

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

INTRODUCTION

The goal is to develop vaccination regimen to a tumor associated self-antigen, ErbB-2 and to induce significant anti-tumor immunity in mice which are tolerant to ErbB-2. The objectives are to

(1) Measure vaccination efficacy after depletion of $CD4^+CD25^+$ cells regulatory T (Treg) cells.
(2) Compare immune reactivity and tumor growth inhibition in ErbB-2 transgenic mice vaccinated with autologous or heterologous ErbB-2
(3) Construct and test vaccination efficacy of ErbB-2 containing Pan DR Reactive Epitope (PADRE).

BODY

(1) Measure vaccination efficacy after depletion of $CD25^+CD4^+$ T reg cells.

Immunization with DNA encoding tumor-associated antigen has demonstrated striking efficacy in mice. Many tumor-associated antigens are, however, normal molecules over-expressed or inappropriately expressed. There may exist significant tolerance to these tumor-associated antigens. One of the most promising approaches to overcome such tolerance involves the modulation of $CD4^+25^+$ regulatory T (Treg) cells which may suppress anti-tumor immunity.

The effect of Treg cell depletion was first tested on the growth of D2F2/E2 tumor cell line which express human ErbB-2 (E2, Her-2). BALB/c mice were injected i.p. with CD25 mAb on two consecutive days. Depletion of of $CD4^+25^+$ cells was verified by flow cytometry. On day 5 after the second mAb injection when $CD4^+25^+$ T cells were at a minimum, mice were injected s.c. with $2x10^5$ D2F2/E2 cells. In untreated mice, D2F2/E2 tumors were palpable in one to three weeks after inoculation, and they grew to ~ 500 mm^3 in six weeks when the mice were sacrificed (Figure 1). Following Treg cell depletion, six of eight mice developed palpable tumors in less than two weeks, but the tumors started to regress before they were 50 mm^3 in volume. By week 4, all tumors regressed completely. The mice were monitored for another 10 weeks without any sign of tumor recurrence. The same tumor regression was observed when unmodified D2F2 tumor cells were tested in BALB/c mice (not shown).

The dramatic increase in anti-tumor immunity was induced by simply depleting Treg cells. This induction of functional immunity to a growing tumor, i.e. *in situ* priming, challenges the long-standing paradigm that solid tumors are poor at priming the immune system and cannot be rejected effectively by immune cells. Rather, their immunogenicity is thwarted by the regulatory mechanisms.

There is a finite window in which CD25 mAb can be used to selectively deplete Treg cells because activated T cells also express CD25 and are sensitive to this antibody. It would be detrimental if CD25 mAb is administered after effector T cell activation. It may be possible to further amplify the striking anti-tumor immunity with reagents like GITR antibody which removes Treg cell activity without depleting effector cells. These are powerful tools and there is significant concern that such treatment can open the gate

to autoimmunity. To access the extent of this possibility, we have chosen autoimmune thyroiditis as a prototype autoimmune disease to gauge the potential complications

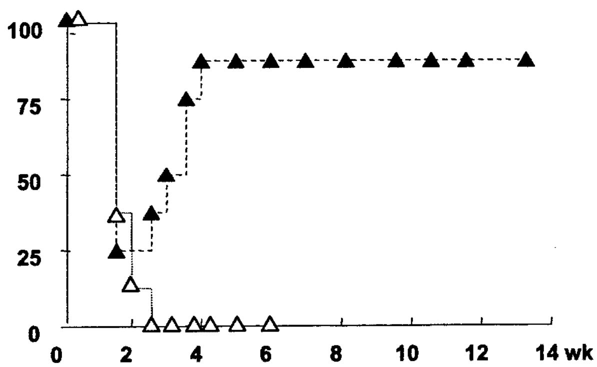


Figure 1 Tumor regression following $CD4^+25^+$ T cell depletion. BALB/c mice were injected i.p. with anti-CD25 mAb PC61 (filled symbol) 5 and 6 day before they received s.c. $2x10^5$ D2F2/E2 cells. Control mice were treated with normal sera or PBS (open symbol). There were eight mice each in PC61 treated and control groups.

Autoimmune thyroiditis was among the first autoimmune complications recognized in the mid-1970s, following the removal of putative suppressor T cells in rodents. Experimental autoimmune thyroiditis (EAT) has served as a prototype autoimmune disease model because it is inducible with a self antigen, mouse thyroglobulin (mTg), a homologue of human thyroglobulin, and shares well-delineated features with human Hashimoto's thyroiditis (HT),

The potential of using EAT as a prototypical autoimmune disease to assess the effect of Treg cell manipulation was tested by depleting Treg cells in tolerized mice prior to EAT induction. CBA mice which are highly susceptible to EAT were used initially. We first established tolerance with the tolerogenic regimen of two doses of deaggregated mTg (dmTg). Treg cells were then depleted with CD25 mAb. The mice were subsequently challenged with the EAT-inducing regimen of mTg and LPS. Depletion of $CD4^+25^+$ T cells abrogated protection against EAT induction; 100% (6/6) of tolerized mice became susceptible to EAT induction with thyroid damage (thyroid infiltration of 10% or more), compared to 17% (1/6) mice in the tolerized, IgG control group. With the establishment that Treg cells are involved in the immune regulation of EAT-susceptible CBA mice, the role of Treg cells is being tested in relatively EAT-resistant BALB/c strain.

(2) Immune reactivity and tumor growth inhibition in ErbB-2 transgenic mice

Rat neu transgenic mice are interesting and useful, but there is ~10% difference between rat and human ErbB-2 proteins. In the last progress report, we showed that spontaneous tumorigenesis in BALB NeuT mice was inhibited by rat neu, but not Her-2 immunization, demonstrating poor cross reactivity between human and rat ErbB-2 in BALB/c mice. These results indicated that rat neu transgenic mice may be somewhat inadequate for certain studies involving human ErbB-2 vaccines.

Her-2 transgenic (Her-2 Tg) mice were generated with wild type human c-ErbB-2 (Her-2) under whey acidic protein (WAP) promoter. Her-2 Tg mice are tolerant to Her-2 and appropriate for testing Her-2 vaccines. The expression of transmembrane ErbB-2 from WAP-Her-2 cassette and its up-regulation by insulin and hydrocortisone was verified by <u>in vitro</u> transfection. Transgene cassette was microinjected into fertilized eggs from B6C3 (C3H x C57Bl/6) females mated with B6C3 males. Transgene positive mice were back-crossed onto C57Bl/6 mice. Human ErbB-2 was expressed in the

secretory mammary epithelia during pregnancy and lactation and expressed constitutively in the Bergman glia cells within the molecular layer of the cerebellum. Overt, neoplastic transformation was not detected in any tissue examined.

Tolerance to Her-2 was demonstrated by inoculating mice with a syngenic tumor expressing high levels of human ErbB-2. Tumors grew exclusively in Her-2 Tg mice without inducing an antibody response, while the nontransgenic littermates remained tumor free for 10 months and mounted a robust anti-ErbB-2 antibody response. When immunized five times with plasmid DNA encoding secErbB-2 and GM-CSF, respectively, about 33% of the Her-2 Tg mice rejected a lethal challenge of EL-4/E2 tumor cells, whereas all immunized littermates rejected the tumor. Therefore, Her-2 Tg mice express human ErbB-2 in the brain and mammary gland and demonstrated tolerance to ErbB-2 which was partially overcome by DNA vaccination. The breakable tolerance of Her-2 Tg mice resembles that in human and these mice are particularly suited for testing human ErbB-2 based vaccines.

KEY RESEARCH ACCOMPLISHMENTS

1. Establish the role of CD4+25+ Treg cells in anti-ErbB-2 immunity and in the induction of experimental autoimmune thyroiditis (EAT). EAT will be used to assess autoimmune side effects in cancer immunotherapy involving Treg cell modulation.
2. Establish syngeneic C57Bl/6 Her-2 Tg mice which are tolerant to human ErbB-2 and suitable for testing Her-2 based vaccines.

REPORTABLE OUTCOMES (one copy attached)

Marie P. Piechocki, Ye-Shih Ho, Shari Pilon and Wei-Zen Wei, Human ErbB-2 (Her-2) transgenic mice: A model system for testing Her-2 based vaccines, J. Immunol. In Press

Wei-Zen Wei, Gerald P. Morris and Yi-chi M. Kong, Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells. Cancer Immunology and Immunotherapy, In Press

CONCLUSIONS

Depletion of Treg cells results in a window of opportunity for active vaccination against tumor associated antigens. The possible autoimmune side-effect will be monitored by the development of experimental autoimmune thyroiditis. Her-2 tolerant C57Bl/6 Her-2 transgenic mice have been established and will be used to test human Her-2 based vaccines. By addressing these two major obstacles in cancer immunotherapy research, the new test systems will make it possible to evaluate the efficacy and prevent side effects of cancer vaccines.

Autoimmunity and anti-tumor immunity – a balancing act of regulatory T cells

Wei-Zen Wei*, Gerald P. Morris and Yi-chi M. Kong
*Karmanos Cancer Institute, Department of Immunology and Microbiology
Wayne State University, Detroit, Michigan 48201

The quest to control cancer by immunological means has met with only occasional success. There has been much speculation on this inadequacy and many studies have been designed to delineate this difficulty[1-3]. For example, tumor cells may lose class I major histocompatibility antigen (MHC I), disabling their recognition by cytotoxic T cells[1,4]. They may produce immunosuppressive molecules like TGF-β[5,6] to dampen immune stimulation. Solid tumors may be encapsulated, posing a physical barrier to immune cells[7]. To overcome these obstacles and elicit a functional anti-tumor immunity, a variety of strategies have been proposed. One of the most promising approaches involves the modulation of regulatory T (Treg) cells which may inhibit the induction of anti-tumor immunity.

Immune regulatory or suppressor activity was observed in many laboratories in the 1970s[8-10]. An elaborate T cell suppressor pathway was proposed to illustrate how suppressor cells and factors inhibit anti-tumor immunity[11,12]. It was suggested that tumor antigens preferentially activate the suppressor pathway[12], and that cyclophosphamide abrogates some, but not all, suppressive mechanisms[11]. At the time, the suppressor cells and factors were characterized mainly by their functions, since they were difficult to isolate and molecular markers were unavailable. Further studies regarding the nature of suppressor cells merely raised questions of their very existence and the pursuit of suppressor cells was abandoned in most labs. With new molecular markers, suppressor-like or regulatory cells have been thrust into new light. Both myelocytic[13,14] and lymphocytic regulatory cells have been described. The discussion here will be focused on Treg cells.

Regulatory T (Treg) cells in autoimmunity

Similar to suppressor cells in anti-tumor immunity, suppressor cells in autoimmune diseases were described in the mid-1970s. In both the mouse[15] and rat[16], a low incidence of autoimmune thyroiditis arose spontaneously after postnatal thymectomy and irradiation to deplete putative suppressor T cells. A decade later, co-transfer studies in nude mice showed a T cell subset capable of suppressing the emergence of autoimmune diseases, such as gastritis, oophoritis, orchitis, as well as thyroiditis[17]. Confirming the important role of Treg cells, we demonstrated in a murine model of experimental autoimmune thyroiditis (EAT) that Treg cells were induced in 2-3 days after tolerance induction with soluble mouse thyroglobulin (mTg) and that subsequent induction of EAT was prevented[18]. These regulatory cells were $CD4^+$ [19]. The rapid induction of regulatory activity suggested the amplification of an existing Treg population mediating natural tolerance. The recent identification of the CD25 (IL-2 receptor α) marker on cells with regulatory activity has permitted further characterization of Treg cells mediating natural tolerance. Whereas the transfer of $CD25^-$ cells into nude mice resulted in autoimmune diseases, including thyroiditis and gastritis, $CD25^+$ cells blocked such development[20]. Similarly, absence of $CD4^+25^+$ Treg activity led to autoimmune gastritis[21] and transfer of Treg cells delayed diabetes in CD28-deficient NOD mice[22]. $CD4^+25^+$ T cell was thus recognized as a candidate Treg cell in autoimmunity.

Characterization of Treg cells

Thymus-derived $CD25^+$ T cells represent ~10% of peripheral $CD4^+$ T cells and encompass a major Treg cell activity. However, CD25 is expressed on both Treg and effector T cells. *In vivo* treatment of mice with a CD25 mAb, PC61, depletes Treg cell activity in naïve mice, but depletes both Treg and effector T cells in immunized mice. Other markers associated with $CD4^+25^+$ Treg cells include CTLA-4[23,24], $CD45RB^{low}$[23,25], and Toll-like receptors 4, 5, 7 and 8[26]. Recently, two additional markers have shown high correlation with Treg activity. One is glucocorticoid-induced tumor necrosis factor receptor family–related gene (GITR)[27]. Similar to CD25, elevated GITR is expressed on both Treg and activated T cells[28]. GITR is a 228 a.a., cysteine rich, type 1 transmembrane protein of the TNF family[29] and forms homodimers on the cell surface. A non-depleting GITR mAb, DTA-1, triggers T cell signaling and abolishes suppressive activity without depleting cells[28]. Systemic treatment of BALB/c mice with GITR mAb resulted in spontaneous gastritis[28], indicating the induction of autoimmunity by functionally disabling Treg cells.

The most specific marker of Treg cells to date is scurfin, a transcription factor encoded by forkhead box P3 gene Foxp3[30-33]. Scurfin binds to the promoter region of cytokine genes and attenuates the production of activation-induced cytokine, such as IL-2[30], keeping Treg cells from proliferation when stimulated. When transduced with Foxp3 gene or isolated from Foxp3 transgenic mice, even $CD4^+25^-$ T cells exerted suppressive activity[31,33]. Conversely, Foxp3 knock-out mice developed lymphoproliferative disease, similar to Foxp3-mutant Scurfy mice or CTLA-4[34] deficient mice, further supporting the association of Foxp3 with negative regulation[32]. Over-expression of Foxp3 in CTLA-4 deficient mice delayed lymphoproliferative disease[33], compensating for the lack of CTLA-4 function. These findings support $CD4^+25^+Foxp3^+$ cells as a distinct lineage of Treg cells.

Functionally, Treg cells exert suppressive activity in a non-specific fashion when their TCRs are triggered[35]. Cell-cell contact appears necessary[35] and membrane-bound TGF-β may contribute to the activity[23]. This function may suppress self-reactive T lymphocytes that escape thymic clonal deletion[36,37] or prevent over-reaction to pathogens[38]. Other T cells may exist and regulate immune function through IL-10, but they may represent cell populations distinct from $Foxp3^+$ Treg cells[39].

Treg cell depletion on anti-tumor immunity and the induction of autoimmunity

Treg-like $CD4^+25^+$ T cells with suppressive activity have been described in lung[40], pancreas and breast cancer patients[41], although it is unclear if these cells are of Foxp3 lineage. In mice, liberation from negative regulation by CD25 mAb treatment led to regression of leukemia and fibrosarcoma[42]. In another study, depletion of $CD25^+$ Treg cells resulted in lower incidence or slower growth rate of B16F10 tumor[43]. Both $CD4^+$ and $CD8^+$ T cells contributed to this anti-tumor activity. Priming to B16F10 associated antigens was evidenced by B16F10 tumor rejection in naïve mice which received adoptively transferred $CD4^+$ T cells. Immune priming was further demonstrated by specific recognition of a self antigen, tyrosinase, in mice which rejected B16F10 tumor with CD25 mAb treatment.

The amplification of anti-tumor immunity through Treg cell modulation may be complicated by immune reactivity to non-tumor associated, self-antigens. In 14 patients with metastatic melanoma, there were two compete and one partial responders following treatment with human mAbMDX-010 for blocking CTLA-4 engagement to B7 and s.c. vaccination with two modified HLA-A*0201-restricted peptides[44]. In six patients (43%), grade III/IV

autoimmune manifestations were observed, including dermatitis, enterocolitis, hepatitis and hypophysitis. The three patients with objective cancer regression all developed severe autoimmune symptoms requiring treatment. The trial with a two-stage design was intended to accrue 21 patients in the first stage. The accrual was ceased after 14 patients because of these autoimmune complications.

By modulating Treg cells, cancer immunotherapy is encountering an unprecedented opportunity, yet with a clear and present danger. Although autoimmunity is being handled in patients as it arises, there is an urgent need to analyze in a comprehensive manner the two sides of Treg cell modulation. New and innovative strategies must be developed to implement cancer control without falling prey to autoimmunity. Toward this end, animal model systems to access simultaneously anti-tumor and autoimmunity following Treg cell modulation have to be established.

Treg cell depletion on tumor growth in BALB/c mice

Many tumor models have been developed in BALB/c mice. Thus, the effect of Treg cell depletion on tumor growth was tested in these mice using D2F2/E2 tumor cell line. D2F2 is a mouse mammary tumor line derived from a spontaneous mammary tumor that arose in a BALB/c hyperplastic alveolar nodule (HAN) line D2 originally induced by prolactin stimulation[45,46]. To introduce a defined tumor-associated antigen into the cell line, D2F2 cells were transfected with pCMV/E2 which encodes wild-type human ErbB-2 (Her-2) and a stable D2F2/E2 line was generated[45]. Human ErbB-2 (Her-2) is over-expressed in breast, ovarian and several other cancer types. In stage IV breast cancer patients, therapeutic efficacy of Her-2 mAb, Herceptin, has been demonstrated. Her-2 based vaccines, including Her-2 DNA vaccine generated in our lab, are being tested in clinical trials. In human ErbB-2 DNA immunized mice, rejection of D2F2/E2 tumor was mediated primarily by T cells, although significant humoral response was induced[47,48].

BALB/c mice were injected i.p. with CD25 mAb on two consecutive days. Depletion of of $CD4^+25^+$ cells was verified by flow cytometry. On day 5 after the second mAb injection when $CD4^+25^+$ $GITR^+$ T cells were at a minimum, mice were injected s.c. with $2x10^5$ D2F2/E2 cells. In untreated mice, D2F2/E2 tumors were palpable in one to three weeks after inoculation, and they grew to ~ 500 mm^3 in six weeks when the mice were sacrificed (Figure 1). Following Treg cell depletion, six of eight mice developed palpable tumors in less than two weeks, but the tumors started to regress before they were 50 mm^3 in volume. By week 4, all tumors regressed completely. The mice were monitored for another 10 weeks without any sign of tumor recurrence. The same tumor regression was observed when unmodified D2F2 tumor cells were tested in BALB/c mice (not shown).

The dramatic increase in anti-tumor immunity was induced by simply depleting Treg cells. This induction of functional immunity to a growing tumor, i.e. *in situ* priming, challenges the long-standing paradigm that solid tumors are poor at priming the immune system and cannot be rejected effectively by immune cells. Rather, their immunogenicity is thwarted by the regulatory pathway.

There is a finite window in which CD25 mAb can be used to selectively deplete Treg cells because activated T cells also express CD25 and are sensitive to this antibody. It would be detrimental if CD25 mAb is administered after effector T cell activation. It may be possible to further amplify the striking anti-tumor immunity with reagents like GITR antibody which removes Treg cell activity without depleting effector cells. These are powerful tools and there is

significant concern that such treatment can open the gate to autoimmunity. To access the extent of this possibility, we have chosen autoimmune thyroiditis as a prototype autoimmune disease to gauge the potential complications

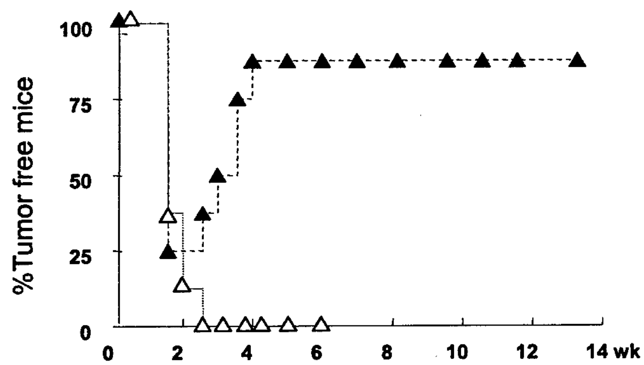


Figure 1 Tumor regression following CD4⁺25⁺ T cell depletion. BALB/c mice were injected i.p. with anti-CD25 mAb PC61 (filled symbol) 5 and 6 day before they received s.c. $2x10^5$ D2F2/E2 cells. Control mice were treated with normal mouse sera or PBS (open symbol). There were eight mice each in PC61 treated and control groups.

Thyroiditis and Treg cells.

Autoimmune thyroiditis was among the first autoimmune complications recognized in the mid-1970s, following the removal of putative suppressor T cells in rodents[15,16]. Because it is inducible with a self antigen, mouse thyroglobulin (mTg), a homologue of human thyroglobulin, in the presence of strong bacterial adjuvant and shares well-delineated features with human Hashimoto's thyroiditis (HT), experimental autoimmune thyroiditis (EAT) has served as a prototype autoimmune disease model[49,50]. HT is a well-characterized, organ-specific disease with known thyroid antigens. Similar to EAT with susceptibility linked to the mouse MHC class II genes, HT susceptibility is strongly influenced by the human MHC class II genes, such as HLA-DRB1*0301, supplemented by CTLA-4 genes in disease development[51,52]. Clinically, HT is an easily diagnosed hypothyroid syndrome, characterized by elevated thyroid-stimulating hormone (TSH) and decreased thyroid hormone levels. This syndrome results from destruction of the thyroid gland by mononuclear cell infiltration[53]. Early autoimmune responses can be monitored by autoantibody production and T cell proliferative response *in vitro* to thyroid antigens. Although HT progresses chronically, it can be managed by thyroid hormone replacement therapy.

The role of Treg cells in EAT tolerance has long been recognized. Treg activity in EAT can be specifically enhanced by elevating the circulatory mTg level for 2-3 days, either by pretreating susceptible mice with mTg[18], as mentioned above, or by TSH infusion to release endogenous mTg[54]. Subsequent induction of EAT with mTg and adjuvant such as complete Freund's adjuvant or lipopolysaccharide is markedly suppressed in such tolerized mice. Tolerance is mediated by $CD4^+$ Treg cells[19] which exert their suppressive action for >10 weeks[55]. The interval of 2-3 days for TCR engagement with elevated mTg antigen is evidently sufficient for the activation and expansion of Treg cells, such that the animals can withstand EAT-induction with mTg plus an adjuvant. It is important to note that immunization with mTg plus proinflammatory cytokines, such as IL-1[56] or IL-12[57], was not sufficient to overcome this tolerant state. We have ruled out Th2-related influence on Treg activity, since IL-4 and IL-10, separately or together, did not participate in mTg-induced tolerance[58].

Treg cell depletion on thyroiditis

The potential of using EAT as a prototypical autoimmune disease to assess the effect of Treg cell manipulation was tested by depleting Treg cells in tolerized mice prior to EAT induction. EAT susceptible CBA mice were used initially. We first established tolerance with the tolerogenic regimen of two doses of deaggregated mTg (dmTg). Treg cells were then depleted with two doses of CD25 mAb, 4 days apart. Depletion of $CD4^{+}25^{+}$ cells was verified as before. The mice were subsequently challenged with the EAT-inducing regimen of mTg and LPS. Depletion of $CD4^{+}25^{+}$ T cells abrogated protection against EAT induction; 100% (6/6) of tolerized mice became susceptible to EAT induction with thyroid damage (thyroid infiltration of 10% or more), compared to 17% (1/6) mice in the tolerized, IgG control group (Figure 2).

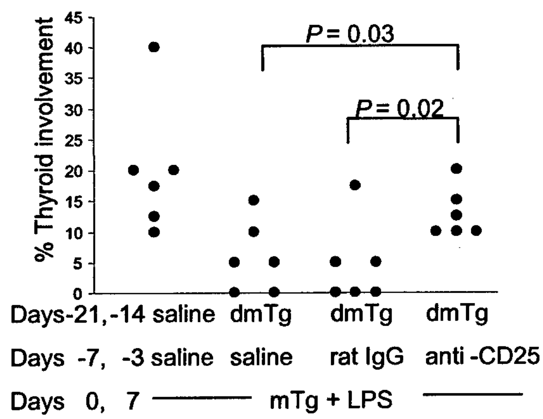


Figure 2 *In vivo* depletion of $CD4^{+}25^{+}$ T cells in mice with established tolerance abrogates protection against EAT. Mice were given 100µg deaggregated mTg or saline, followed by anti-CD25 or IgG control as shown. EAT was induced with 40µg mTg + 20µg LPS, and thyroid pathology was examined on day 28.

With the establishment that Treg cells are involved in the immune regulation of EAT-susceptible CBA mice[59], the role of Treg cells was tested in relatively EAT-resistant BALB/c strain. In a pilot experiment, anti-CD25 treatment prior to EAT induction led to marked thyroid damage in 100% of the mice, compared to mild inflammation in 43% of control mice (data not shown). Therefore, Treg cells are critical in maintaining EAT resistance in BALB/c mice and these mice are well suited for assessing the role of Treg cells and the effect of Treg cell modulation on autoimmune thyroiditis.

Tolerance to tumor-associated antigens

The pressing need to modulate Treg cells stems from the observed tolerance to tumor associated antigens. Although profound anti-tumor immunity to tumors like D2F2/E2, which contains a foreign human ErbB-2 antigen, can be readily induced in BALB/c mice by one-time, transient depletion of Treg cells (Figure 1), the same may not be true for an over-expressed self antigen in human cancer. More strenuous Treg cell modulation may be required to achieve therapeutic effect, creating even greater risk of autoimmunity[44]. A test system with tolerance to tumor-associated antigen, e.g. ErbB-2, will be more appropriate for assessing the effect of Treg cell modulation, and BALB NeuT (NeuT) mice are particularly suited for this purpose. NeuT mice express a transforming rat Neu under MMTV promoter and have been back-crossed to BALB/c mice for >12 generations[60]. NeuT females develop atypical hyperplasia, carcinoma *in situ* and palpable mammary tumors at ~5, 10 and 17 wk of age, respectively. Males develop salivary tumors at ~7 months of age. Spontaneous tumors express abundant transgenic neu. These NeuT mice are tolerant to Neu and respond weakly to Neu vaccination[61]. Blockade of

spontaneous tumorigenesis in NeuT mice is a significant challenge, particularly after carcinoma has been well established *in situ*.

In our lab, transgenic mice expressing wild type human ErbB-2 (Her-2 Tg) have been established[62] and have been back-crossed with C57BL/6 mice for >12 generations. These mice do not develop spontaneous tumor, but respond poorly to human ErbB-2 vaccination. Treg cell modulation in BALB NeuT or C57BL/6 Her-2 Tg mice to control ErbB-2 positive tumors will provide an appropriate setting for assessing tolerance to tumor-associated antigens and potential autoimmune complications.

Balancing anti-tumor and autoimmunity when manipulating Treg cells

New strategies to induce strong anti-tumor immunity without significant autoimmunity will be the next milestone in tumor immunotherapy. To achieve anti-tumor immunity, it will be important to eliminate Treg cells to tumor-associated antigen. To avoid autoimmunity, it is advantageous to amplify Treg cells to self-antigens. The available tools for manipulating Treg cells, such as mAb to CD25, GITR and CTLA-4, do not distinguish one Treg cell from another. Will these tools be useful in cancer therapy ? Perhaps the first order of business is to determine whether all Treg cells in a given individual are created equal and therefore, equally sensitive to these modulating agents. Using thyroiditis as a model system with known MHC class II-based susceptibility, one can test if Treg cells generated during thyroglobuin-induced tolerance are qualitatively different from resident Treg cells in naïve mice. Alternatively, it may be advantageous to inactivate locally tumor-specific Treg cells at the vaccination site to amplify priming to tumor-associated antigens without systemic down-modulation of Treg cells, thus minimizing the priming to self-antigens. The insight on Treg cells, the available molecular tools and the awareness of autoimmune complications will guide the renewed quest for cancer immunotherapy.

Reference List

(1) Seliger B, Ritz U, Abele R et al. Immune Escape of Melanoma: First Evidence of Structural Alterations in Two Distinct Components of the MHC Class I Antigen Processing Pathway. Cancer Res. 2001;61:8647-8650.

(2) Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nature Immunology. 2002;3:991-998.

(3) Khong HT, Restifo NP. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. Nature Immunology. 2002;3:999-1005.

(4) Ferrone S, Marincola FM. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. Immunology Today. 1995;16:487-494.

(5) Piek E, Roberts AB. Suppressor and oncogenic roles of transforming growth factor-beta and its signaling pathways in tumorigenesis. Advances in Cancer Research. 2001;83:1-54.

(6) Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. Nature Medicine. 2001;7:1118-1122.

(7) Spiotto MT, Yu P, Rowley DA et al. Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells. Immunity. 2002;17:737-747.

(8) Calkins C, Stutman O. Changes in suppressor mechanisms during postnatal development in mice. Journal of Experimental Medicine. 1978;147:87-97.

(9) Higert I. The involvement of activated specific suppressor T cells in maintenance of transplantation tolerance. Immunology Review. 1979;46:27-53.

(10) Asherson G, Zembala M, Thomas W, Perera M. Suppressor cells and the handling of antigen. Immunology Review. 2003;50:3-45.

(11) Schatten S, Drebin JA, Perry LL, Chung W, Greene MI. Regulation of the immune response to tumor antigens. X. Activation of third-order suppressor T cells that abrogate anti-tumor immune responses. Journal of Immunology. 1984;133:1064-1069.

(12) Schatten S, Granstein RD, Drebin JA, Greene MI. Suppressor T cells and the immune response to tumors. Crit Rev Immunol. 1984;4:335-379.

(13) Pekarek LA, Starr BA, Toledano AY, Schreiber H. Inhibition of tumor growth by elimination of granulocytes. Journal of Experimental Medicine. 1995;181:435-440.

(14) Young MR, Wright MA, Pandit R. Myeloid differentiation treatment to diminish the presence of immune-suppressive CD34+ cells within human head and neck squamous cell carcinomas. The Journal of Immunology. 1997;159:990-996.

(15) Kojima A, Tanaka-Kojima Y, Sakakura T, Nishizuka Y. Spontaneous development of autoimmune thyroiditis in neonatally thymectomized mice. Lab Invest. 1976;34:550-557.

(16) Penhale WJ, Farmer A, Irvine WJ. Thyroiditis in T cell-depleted rats: influence of strain, radiation dose, adjuvants and antilymphocyte serum. Clin Exp Immunol. 1975;21:362-375.

(17) Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. Journal of Experimental Medicine. 1985;161:72-87.

(18) Kong YM, Okayasu I, Giraldo AA et al. Tolerance to thyroglobulin by activating suppressor mechanisms. Annals of New York Academy of Science. 1982;392:191-209.

(19) Kong YM, Giraldo AA, Waldmann H, Cobbold SP, Fuller BE. Resistance to experimental autoimmune thyroiditis: L3T4+ cells as mediators of both thyroglobulin-activated and TSH-induced suppression. Clin Immunol Immunopathol. 1989;51:38-54.

(20) Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. Journal of Immunology. 1995;155:1151-1164.

(21) Scheinecker C, McHugh R, Shevach EM, Germain RN. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. Journal of Experimental Medicine. 2002;196:1079-1090.

(22) Salomon B, Lenschow DJ, Rhee L et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity. 2000;12:431-440.

(23) Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated Antigen 4 plays an essential role in the function of $CD25^{+}CD4^{+}$ regulatory cells that control intestinal inflammation. Journal of Experimental Medicine. 2000;192:295-302.

(24) Takahashi T, Tagami T, Yamazaki S et al. Immunologic self-tolerance maintained by $CD25^{+}CD4^{+}$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. Journal of Experimental Medicine. 2000;192:303-309.

(25) Itoh M, Takahashi T, Sakaguchi N et al. Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. The Journal of Immunology. 1999;162:5317-5326.

(26) Caramalho I, Lopes-Carvalho T, Ostler D et al. Regulatory T cells express toll-like receptors and are activated by lipopolysaccharide. Journal of Experimental Medicine. 2003;197:403-411.

(27) Shimizu J, Yamazaki S, Takahashi T et al. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. Nature Immunology. 2002;3:135-142.

(28) Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. Nature Immunology. 2002;2:135-142.

(29) Nocentini G, Giunchi L, Ronchetti S et al. A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. Proc Natl Acad Sci. 2002;94:6216-6221.

(30) Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. The Journal of Biological Chemistry. 2001;267:37672-37679.

(31) Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299:1057-1061.

(32) Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4(+)CD25(+) regulatory T cells. Nature Immunology. 2003;4:330-336.

(33) Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4(+)CD25(+) T regulatory cells. Nature Immunology. 2003;4:337-342.

(34) Tivol EA, Borriello F, Schweitzer AN et al. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3:541-547.

(35) Thornton AM, Shevach EM. Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. Journal of Immunology. 2000;164:183-190.

(36) Shevach EM. Certified professionals: $CD4^{+}$ $CD25^{+}$ suppressor T cells. Journal of Experimental Medicine. 2001;193:F41-F45.

(37) Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nature Reviews Immunology. 2002;2:398-400.

(38) Sakaguchi S. Control of immune responses by naturally arising CD4+ regulatory T cells that express toll-like receptors. Journal of Experimental Medicine. 2003;197:397-401.

(39) Levings MK, Sangregorio R, Sarukhan A et al. Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. Journal of Experimental Medicine. 2002;196:1335-1346.

(40) Woo EY, Yeh H, Chu CS et al. Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. The Journal of Immunology. 2002;168:4272-4276.

(41) Liyanage UK, Moore TT, Joo HG et al. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. The Journal of Immunology. 2002;169:2756-2761.

(42) Shimizu J, Yamazaki S, and Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. The Journal of Immunology. 1999;163:5211-5218.

(43) Jones E, Dahm-Vicker M, Simon AK et al. Depletion of CD25+ regulatory cells results in suppression of melanoma growth and induction of autoreactivity in mice. Cancer Immunity. 2002;2:1.

(44) Phan GQ, Yang JC, Sherry RM et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci. 2003;100:8372-8377.

(45) Wei WZ, Shi WP, Galy A et al. Protection Against Mammary Tumor Growth By Vaccination With Full-Length, Modified Human *ErbB-2* DNA. Int J Cancer. 1999;81:748-754.

(46) Mahoney KH, Miller BE, Heppner GH. FACS quantitation of leucine aminopeptidase and acid phosphatase on tumor associated macrophages from metastatic and nonmetastatic mouse mammary tumors. Journal of Leukocyte Biology. 1985;38:573-585.

(47) Piechocki MP, Pilon S, Wei W-Z. Complementary antitumor immunity induced by plasmid DNA encoding secreted and cytoplasmic human ErbB-2. The Journal of Immunology. 2001;167:3367-3374.

(48) Pilon S, Piechocki MP, Wei W-Z. Vaccination with cytoplasmic ErbB-2 DNA protects mice from mammary tumor growth without anti-ErbB-2 antibody. The Journal of Immunology. 2001;167:3201-3206.

(49) Vladutiu AO, Rose NR. Autoimmune murine thyroiditis: relation to histocompatibility (H-2) type. Science. 1971;174:1137-1139.

(50) Kong YM. Experimental models for autoimmune thyroid disease: recent developments. In: Volpe R, ed. Contemporary endocrinology: autoimmune endocrinopathies. Totowa, NJ: Humana Press Inc.; 1999:91-111.

(51) Vaidya B, Kendall-Taylor P, Pearce SHS. Genetics of endocrine disease: the genetics of autoimmune thyroid disease. J Clin Endocrinol Metab. 2002;87:5385-5397.

(52) Ueda H, Howson J, Esposito L, et.al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature. 2003;423:506-511.

(53) Volpé R. Autoimmune diseases of the endocrine system. 1st ed. Boca Raton: CRC Press; 1990.

(54) Lewis M, Giraldo AA, Kong YM. Resistance to experimental autoimmune thyroiditis induced by physiologic manipulation of thyroglobulin level. Clin Immunol Immunopathol. 1987;45:92-104.

(55) Fuller BE, Okayasu I, Simon LL, Giraldo AA, Kong YM. Characterization of resistance to murine experimental autoimmune thyroiditis: duration and afferent action

of thyroglobulin- and TSH-induced suppression. Clin Immunol Immunopathol. 1993;69:60-68.

(56) Nabozny GH, Kong YM. Circumvention of the induction of resistance in murine experimental autoimmune thyroiditis by recombinant IL-1β. The Journal of Immunology. 1992;149:1086-1092.

(57) Zhang W and Kong YM. IL-12 interferes with tolerance induction to experimental autoimmune thyroiditis [abstract]. FASEB J. 1998;12:A1096.

(58) Zhang W, Kong YM. Noninvolvement of IL-4 and IL-10 in tolerance induction to experimental autoimmune thyroiditis. Cellular Immunology. 1998;187:95-102.

(59) Morris GP, Chen L, and Kong YM. CD137 signaling interferes with activation and function of regulatory $CD4^{+}CD25^{+}$ T cells in induced tolerance to experimental autoimmune thyroiditis (EAT) [abstract]. FASEB J. 2003;17:C258.

(60) Boggio K, Nicoletti G, Di Carlo E et al. Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/*neu* transgenic mice. Journal of Experimental Medicine. 1996;188:589-596.

(61) Rovero S, Amici A, Di Carlo E et al. Inhibition of carcinogenesis by DNA vaccination. The Journal of Immunology. 2000;165:5133-5142.

(62) Piechocki MP, Ho YS, Wei WZ. Transgenic mice expressing human ErbB-2 (E2) under whey acidic protein promoter exhibit immunological tolerance to E2. Proceedings of AACR. [abstract] 2001;42:560.

Human ErbB-2 (Her-2) transgenic mice:

A model System for testing Her-2 based Vaccines[1]

Marie P. Piechocki[*,†], Ye-Shih Ho[‡], Shari Pilon[*,§] and Wei-Zen Wei[*,§,2]

Wayne State University, School of Medicine, Karmanos Cancer Institute[*] Department of Otolaryngology[†], Institute of Environmental Health Sciences[‡], Department of Immunology and Microbiology[§], Detroit, MI

[2]Address correspondence and reprint requests to

Wei-Zen Wei, Ph.D.

Karmanos Cancer Institute

110 E. Warren Avenue

Detroit, MI 48201

Phone: 313-833-0715

Fax: 313-832-4537

Email: weiw@karmanos.org

Running Title: Human ErbB-2 transgenic mice

Key Words: Tumor immunity, Vaccination, Transgenic/knockout, rodent, human

Word Count: 6897

[1] This work was supported by NIH CA76340, DOD BC000741, 5 T32 DC00026 (MPP), P30 ES06639, and Gail Purtan fund.

Abstract

Her-2 transgenic (Tg) mice were generated with wild type human c-ErbB-2 (Her-2) under whey acidic protein (WAP) promoter. They are tolerant to Her-2 and appropriate for testing Her-2 vaccines. The expression of transmembrane ErbB-2 from WAP-Her-2 cassette and its up-regulation by insulin and hydrocortisone was verified by in vitro transfection. Transgene cassette was microinjected into fertilized eggs from B6C3 (C3H x C57Bl/6) females mated with B6C3 males. Transgene positive mice were back-crossed onto C57Bl/6 mice. Human ErbB-2 was expressed in the secretory mammary epithelia during pregnancy and lactation and expressed constitutively in the Bergman glia cells within the molecular layer of the cerebellum. Overt, neoplastic transformation was not detected in any tissue examined. Tolerance to Her-2 was demonstrated by inoculating mice with a syngenic tumor expressing high levels of human ErbB-2. Tumors grew exclusively in Her-2 Tg mice without inducing an antibody response, while the nontransgenic littermates remained tumor free for 10 months and mounted a robust anti-ErbB-2 antibody response. When immunized five times with plasmid DNA encoding secErbB-2 and GM-CSF, respectively, about 33% of the Her-2 Tg mice rejected a lethal challenge of EL-4/E2 tumor cells, whereas all immunized littermates rejected the tumor. Therefore, Her-2 Tg mice express human ErbB-2 in the brain and mammary gland and demonstrated tolerance to ErbB-2 which was partially overcome by DNA vaccination. The breakable tolerance of Her-2 Tg mice resembles that in human and these mice are particularly suited for testing human ErbB-2 based vaccines.

Introduction

ErbB-2 is amplified in about 30% of all breast cancers and is over-expressed in several other epithelial-derived neoplasms including ovarian cancer, small cell lung cancer and cancers of the head and neck (1-3). The presence of ErbB-2 specific T cells and antibodies in breast and ovarian cancer patients indicated this molecule as a target of immunoprevention and therapy (4-7). Anti-ErbB-2 mAb, Herceptin, is used to treat patients with advanced breast cancer (8). We have generated several human ErbB-2 (Her-2) based DNA vaccines and demonstrated striking anti-tumor immunity in mice (9-11). Since ErbB-2 is a self antigen and the sequence is typically unmodified in human cancer, immune tolerance to this tumor associated antigen is expected in humans. Therefore, the efficacy of ErbB-2 based immunotherapy or vaccines will have to be tested in tolerant hosts.

Several rat ErbB-2 (neu) transgenic mouse strains have been established. FVB-NeuN mice carry wild type rat neu driven by the MMTV promoter in the H-2^q FVB background. Overexpression of the transgene in the mammary gland results in mammary tumor growth around 40 wks of age (12). BALB NeuT mice carry activated rat neu oncogene driven by the MMTV promoter in the H-2^d BALB/c background (13). Spontaneous mammary tumors appear in BALB NeuT females around 20 wks of age. Although these models are interesting and useful, there is ~10% difference between rat and human ErbB-2 proteins (14). ***When immunized with human ErbB-2 DNA, FVB-NeuN females developed less tumors, but neither humoral nor cellular immunity to rat neu was detected and there remained uncertainty if tolerance to rat neu was overcome (15). These results indicated that rat neu transgenic mice may be somewhat inadequate for testing human ErbB-2 vaccines.***

The development of human ErbB-2 transgenic mice was previously attempted with a DNA sequence comprising the promoter-enhancer region of the MMTV-LTR and a constitutively activated allele of the human ErbB-2 (16). Expression of the transgene was

observed in kidney, lung, mammary gland, salivary gland and in the male reproductive track. All transgenic mice expressing ErbB-2 died within four month of age, probably due to kidney and lung failure following the development of preneoplastic lesions. Mammary glands in parous females were underdeveloped and some gave rise to tumors. Transgenic males were sterile.

To generate a new model of human ErbB-2 transgenic mice, we have chosen the whey acidic protein (WAP) promoter. Whey acidic protein is a major whey protein secreted in rodent's milk and WAP promoter has been used for targeting heterologous genes to the mammary gland. Although typically described as being under strict hormonal and lactational control, the expression of whey acidic protein transgenes in normal mice has also been observed in other organs, especially in the brain (17). Here we report the generation of Her-2 transgenic mice using a WAP promoter regulated c-ErbB-2 transgene and the immune tolerance in these mice.

Materials and Methods

Construction of pWAP-human ErbB-2 (pWAP-Her-2)

The full-length human c-ErbB-2 cDNA was isolated from plasmid pCMV-ErbB-2 (9) as a 4.4 kb EcoRI restriction fragment and cloned into the KpnI site downstream of the 2.5 kb WAP promoter in pBSK kindly provide by Dr. Bernd Groner (Chemotherapeutisches Forschungsinstitut, Frankfurt, Germany). E. Coli. DH5α (Life Technologies, Gaithersburg, MD) or Top10 (InVitrogen, Carlsbad, CA) was used to propagate pWAP-Her-2, using Terrific Broth (Life Technologies, Gaithersburg, MD) containing 100 μg/ml of ampicillin. Plasmid was purified with QIAfilter Giga Kit (QIAGEN Inc., Valencia, CA). The 6.9 kb WAP-Her-2 expression cassette was liberated by HinDIII and used for transfection and microinjection.

Mice and Cell lines

Male and Female C57Bl/6 (H2-K^b) mice (6-8 wks of age) were obtained from Charles River Laboratory (Frederick, MD) or the Jackson Laboratory (Bar Harbor, ME). Mouse mammary tumor (MMT) line D2F2 was derived from a spontaneous mammary tumor which arose in a BALB/c hyperplastic alveolar nodule (HAN) line D2 (18). The cell line was maintained *in vitro* in Dulbecco's modified Eagle's medium (MEM) supplemented with 5% heat inactivated fetal bovine serum (Sigma, St. Louis, MO) and 5% Cosmic Calf Serum (HyClone, Logan, UT), 10% NCTC 109 medium (Sigma, St. Louis, MO), 2.5 mM β-mercaptoethanol, 0.5 mM sodium pyruvate, 2 mM L-glutamate, 0.1 mM MEM nonessential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin. ID8/E2 is a C57Bl/6 ovarian cancer cell line (19) transfected with pCMV-ErbB-2. EL-4/E2 is a C57Bl/6 thymoma cell line transfected with pCMV-ErbB-2. All transfected cells were cloned twice by limiting dilution to isolate clones of stable expression. Transfected cell lines were maintained in medium containing 0.8 mg/mL G418 (Geneticin, Sigma).

Expression of WAP-Her-2 cassette in D2F2 cells

D2F2 cells were co-transfected with the 6.9 Kb HinDIII WAP-Her-2 expression cassette and linearized pRSV/neo, at a 10:1 ratio, using LipofectAMINE Plus reagent purchased from GIBCO BRL/Life Technologies (Gaithersburg, MD). Individual colonies were expanded and expression of the recombinant protein was analyzed by flow cytometry, or immunoprecipitation and Western blot. Some of the transfected D2F2 clones were cultured in the presence of 10 μg/ml Insulin and 10 μM hydrocortisone to enhance transcription from WAP promoter.

Flow cytometric analysis

Monoclonal antibodies TA-1 (AB-5) and 3B5 (AB-3) which recognize the extracellular and cytoplasmic domains of ErbB-2, respectively, were purchased from Oncogene Research Products (Cambridge, MA). FITC conjugated goat anti-mouse- IgG was the secondary antibody (Jackson ImmunoResearch Lab., West Grove, PA). Normal mouse Ig or isotype matched mAb were the negative controls. Flow cytometric analysis was performed with a FACSCalibur (Becton Dickinson, Mountain View, CA).

Identification of Her-2 Tg mice by Southern blotting and PCR

For initial identification of Her-2 Tg mice, genomic DNA was isolated from the tail tissues of 3-4 wk old mice using QIAGEN genomic DNA isolation kit (QIAGEN Inc. Valencia, CA) and digested to completion with EcoRI (Life Technologies), transferred to charged Nylon membranes in 20X SSC, cross-linked to the membrane in a UV cross-linker and blocked overnight at 56°C in hybridization buffer (5 X SSC, 0.5% SDS, 5X Denhardt's reagent, 1 μg/mL BSA and supplemented with 10 μg/ml sheared salmon sperm DNA) in a Stratagene rotary hybridization oven.

Southern blot DNA probe was isolated from human ErbB-2 cDNA by EcoRI restriction (Figure 1A). A 1.6 kb DNA fragment encoding ErbB-2 nucleotide 1440 to 3000 was isolated and random primer labeled using the Klenow fragment of DNA polymerase to incorporate radioactive ^{32}P-dCTP (Dupont NEN, 3000 Ci/mmol). The reaction was stopped by the addition of EDTA and the product was purified by passage through a sephadex G-50 column which was prepacked and blocked with salmon sperm DNA (5 Prime-3 Prime, Boulder, CO). Purified probe was denatured by boiling for 5 minutes and snap cooled on ice. Denatured probe was added to hybridization buffer to achieve a final concentration of 2×10^6 cpm per ml. Specific activity of the probe was typically $>10^8$ cpm/μg DNA. Membranes

were hybridized overnight at 56°C and then washed to ultimate stringency (0.1 X SSC, 0.1% SDS, 65 °C). Membranes were sealed in plastic wrap and exposed to Kodak MR-1 film with an intensifying screen for 2-3 days at –80°C.

For routine screening of Her-2 Tg mice, a 2 mm ear punch or tail tissue was collected from 3-4 week-old pups and used for PCR analysis. The tissue was digested in 200 μl of sterile lysis buffer containing 50mM KCl , 10mM Tris-HCl pH 8.3, 2.5 mM $MgCl_2$, 0.2 mg/ml Gelatin (Sigma) and 0.45% v/v IGEPAL CA-630 (Sigma) supplemented with 100 microgram/mL Proteinase K (Sigma P20308). The tissue was digested overnight at 56 °C. Proteinase K was heat-inactivated at 95 °C for 30 minutes. The upper primer 5' CCC CCA CCC CAC CCC CAA AGT C 3' anneals to the whey acidic protein promoter at position –22 relative to the ATG start codon. The lower primer 5' CGG GGG GCA AGA GGG CGA GGA G 3' anneals to the human ErbB-2 cDNA at amino acid 18 downstream of the signal peptide. Amplification of the transgene results in a 352 bp PCR product. Briefly, two to five µl of genomic DNA was amplified in 1X QIAGEN reaction buffer, 200 uM dNTP's, 1 uM of each primer, and 1X Q-buffer in a total volume of 50 uL with 1 unit of Taq polymerase. After an initial denaturation at 94°C for 3 minutes, samples were amplified for 30-35 cycles, consisting of denaturing at 94°C for 1 minute, annealing at 58°C for 1 minute, followed by extension at 72°C for 1 minute. After the last cycle, samples were incubated for 5 minutes at 72°C and resolved in 1.5 % TAE-agarose gels.

Immunoprecipitation and Western blot analysis

Lysates from fresh tissues were prepared by mincing tissues (3 mm^3) in prechilled 1.5 mL microcentrifuge tubes on ice in tissue lysis buffer (50 mM HEPES pH 8.0, 10% glycerol and 1% Triton X-100) supplemented with protease and phosphatase inhibitor cocktails (Oncogene Sciences). Human ErbB-2 protein was immunoprecipitated from the tissue lysates by

incubation with an anti-ErbB-2 mAb 4D5, or Herceptin (Genentech) or mAb 9G10.6 (Neomarkers) for 2-4 hrs. Immune complexes were recovered by incubation with protein A/G Plus agarose (Santa Cruz Biotech) at 4°C for 16-18 hrs. The agarose beads were subjected to centrifugation and washed twice with lysis buffer. Proteins were eluted in 1X sample buffer and boiled for 3 minutes prior to fractionation in 6% SDS-PAGE. Proteins were electrotransferred to Immobilon-P (Millipore, Bedford, MA) PVDF membranes. Membranes were fixed with methanol, rehydrated and blocked overnight at 4°C in TBST buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1% Tween-20) with 1% BSA. ErbB-2 protein was detected by immunoblotting with mAb 3B5 or polyclonal C-18 (Santa Cruz Biotech). Phosphotyrosine was detected with mAb PY20 (Transduction Laboratories). Blots were developed with enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL) and Kodak-MR film.

Immunohistochemical analysis

Tissues were removed from mice and placed immediately in phosphate buffered formalin. Paraffin sections were prepared at 4-5 micron thickness and stained with hematoxylin and eosin. For immunohistochemical analysis, endogenous peroxidase was blocked by incubation in 3% H_2O_2 in methanol or Peroxoblock (Neomarkers). Mild treatment with ficin was used for epitope retrieval. Tissues were processed using the HISTOMOUSE SP kit from Zymed Laboratories (South San Francisco, CA) designed to stain mouse tissues with mouse monoclonal antibodies. The primary antibody TAB250 (Zymed) which recognizes an epitope in the extracellular domain of the human ErbB-2 protein was used according to the recommended procedure. Alternatively, paraffin sections were subjected to HEIR (citrate pH 6.0) and stained with PAD: Z4881 specific for the intracellular domain of human ErbB-2 (Zymed) followed by detection with anti-Rabbit Poly-HRP (Chemicon). Immunostaining was

developed using DAB as the chromagen and nuclei were counterstained with Hematoxylin. Sections were viewed under a Zeiss microscope equipped with a Sony 970 CCD camera and MCID5+ software interface for data acquisition and image analysis with the 25X objective (100X total magnification).

Testing Tolerance and Immunization of Her-2 Tg mice

For plasmid DNA immunization, mice from the 8th generation backcross were injected i.m. with 100 μl of saline containing 100 μg of each component plasmid at two week intervals for a total of five vaccinations. Plasmid DNA expressing full-length and truncated variants of ErbB-2 gene have been described and characterized (9-11). The plasmid pEFBos-GM-CSF encoding murine GM-CSF was provided by Dr. Nishisaki at Osaka University, Osaka, Japan. At two weeks after the final DNA vaccination, mice were challenged s.c. with $2x10^5$ EL-4/E2. Tumors were measured weekly with calipers and animals were sacrificed when any dimension of the tumor exceeded 15 mm. ***The percentage of tumor-free mice was analyzed by Kaplan-Meier method and statistical significance was determined by the log-rank test.***

Measurement of Anti-ErbB-2 antibody

Serum samples were diluted 1:20 and the presence of anti-ErbB2 antibody was determined by flow cytometry using SKBR-3 cells, a human breast carcinoma cell line with amplified ErbB-2. FITC conjugated goat anti-mouse antibody specific for mouse IgG Fcγ (Jackson Immune Research, West Grove, PA) was used to detect bound primary antibody. Normal mouse serum or isotype matched mAb was the negative control. The monoclonal antibody TA-1 (Oncogene Research Products, Cambridge, MA), which recognizes an extracellular domain of ErbB-2, was used as a positive control for detection of ErbB-2 expression on SKBR-3 cells (Oncogene Research Products, Cambridge, MA). Serial dilutions of TA-1 were used to

generate a standard curve to determine the concentration (μg/ml) of anti-ErbB-2 antibody in serum. Flow cytometric analysis was performed with a FACSCalibur (Becton Dickinson, San Jose, CA). Results are presented as concentration, or mean channel fluorescence (MCF). ***Statistical analysis was performed with student's t test.***

Results

Construction and expression of WAP-Her-2

WAP-Her-2 transgene was constructed by fusing the 2.6 kb mouse whey acidic protein (WAP) promoter with the 4.4 kb human ErbB-2 cDNA as described in the Materials and Methods (Figure 1A). Expression of WAP-Her-2 cassette was initially tested by transfecting the 6939 bp fragment into mouse mammary tumor D2F2 cells. Human ErbB-2 protein was detected on the surface of transfected cells by flow cytometry (Figure 1B). Treatment of the transfected cells with insulin and hydrocortisone resulted in nearly a four fold increase in ErbB-2 expression, indicating responsiveness of WAP promoter to the hormone. Immunoprecipitation and Western blot analysis verified the presence of the 185 kD, phosphorylated human ErbB-2 (data not shown).

Purified WAP-Her-2 expression cassette was microinjected into fertilized eggs from B6C3 (C57Bl/6 x C3H F1) females mated with B6C3 males following the procedure described by Hogan et al. (20). The embryos were implanted into pseudopregnant CD-1 surrogate mothers. From 97 live births, seven positive mice were identified by Southern blot analysis using tail tissue lysates. These transgenic founders were bred with C57Bl/6 mice. A total of sixty pups were produced. Seven pups from 4 founders, carried the transgene as detected by Southern blot. These seven pups were regarded as the F1 founders and were mated with C57Bl/6 mice to produce the 1st generation of back-cross (B1) mice. Transgene

positive B1 male mice were backcrossed with female C57Bl/6 mice and transgene distribution in subsequent offspring followed Medalian rule. The Tg mouse line which had the highest and most consistent Tg expression in the early generations was chosen to establish our colony. All studies described here were performed on the progeny of this line.

For routine screening of transgene expression, PCR primers were designed so that the upper primer annealed to the whey acidic protein promoter and the lower primer annealed to ErbB-2 cDNA. A 351 bp product was diagnostic of WAP- Her-2 gene. Using ear punch tissue from ten pups of the 1st backcross (B1) generation, there was 100% concordance between Southern blotting and PCR analysis (Figure 1C). Therefore, routine screening was performed with PCR.

Tissue distribution of human ErbB-2 in Her-2 transgenic mice

Human ErbB-2 expression in the mammary gland was examined in several Her-2 Tg females of the F1 (founder X C57Bl/6) generation (Figure 2) at days 1-2 of lactation.

The #4 mammary glands were removed from Her-2 Tg females at one to three days after they delivered the first or second litter. Tissue lysates were prepared and immunoprecipitated with mAb Herceptin which recognized an epitope in the extra-cellular domain of human ErbB-2 and blotted with mAb 3B5 which recognized a carboxy terminal epitope. In Her-2 Tg mice from the F1 (lanes 4-5) generation, strong expression of p185 was detected. The same dominant band was detected in tissue lysate without immunoprecipitation (lane 3). Furthermore this female produced two female pups with abundant human ErbB-2 expression in the mammary gland (lanes 6-7). No expression was detectable in the non-transgenic lactating littermates (lane 8).

Transgenic mice were crossed with C57Bl/6 mice for 12 generations to establish Her-2 Tg mice in the C57Bl/6 background. Mammary glands from Her-2 Tg females exhibit the

HER2/Neu "signature" phenotype of branching mammary trees with extension beyond the normal fat pad and lobules arrayed in parallel along the milk line and the main artery that supplies all of the mammary glands.

Expression of human ErbB-2 was sustained in mice backcrossed with C57Bl/6. In Figure 3A, immunoprecipitation (IP) and Western blot was used to detect Human ErbB-2 gene product in the mammary tissue and cerebellum of B11 females on day 17-19 of pregnancy or, post-vaginal plug release. Tissue lysates were immunoprecipitated with monoclonal antibodies: 9G6.10, (Figure 3A, lanes 2 and 4) or Herceptin (lanes 3 and 5) which recognized different epitopes of human ErbB-2. Immunoprecipitated proteins were detected by Western blot using pAb C-18 (Santa Cruz). Similar to F1 mice, p185 was detected in both mammary (lanes 2 and 3) and cerebellum (lanes 4 and 5) tissue lysates. Lane 1 is the cerebellum tissue lysate used for immunoprecipitation analysis in lanes 4 and 5. In Figure 3B, we defined the localization of human ErbB-2 in the mammary gland (a+b) and cerebellum (c+d) using immunohistochemistry. Secretory mammary epithelia lining mature ductules and cross-sections of terminal endbud clusters exhibit intensely positive membrane staining by anti-human ErbB-2 antibody (arrows) while supporting stromal cells and fibroblasts are clearly negative (asterisks). In the cerebellum, intense ErbB-2 was detected in the molecular layer of the cerebellum (Figure 3B, c+d). Distribution of the protein was prominent along the Bergman glia fibers (arrows, see figure legend) in the molecular layer (ML) and to a lesser extent in the membranes of the purkinje cells (PCL), but not detectable in the granular layer (GL). Transgene expression in the brain was also documented in WAP-hGH transgenic mice (21).

ErbB-2 protein expression in Her-2 Tg males was also characterized. As in the females (Figure 3B, c+d), intense human ErbB-2 specific immunoreactivity was observed in the molecular layer of the cerebellum in Her-2 Tg males (Figure 3B, e+f). Human ErbB-2 protein

was detected in the cerebellum by immunoprecipitation and Western Blotting (Figure 3C, lane 3). Human ErbB-2 protein was not detected in the parotid (lane 1), other salivary glands (lane 2) nor cerebrum (lane 4). The same tissues taken from a transgene negative littermate were unequivocally negative (lanes 5-8). We further demonstrated that transgenic ErbB-2 in the cerebellum was indeed phosphorylated on tyrosine residues using an HRP-conjugated monoclonal antibody against phosphotyrosine (Figure 3D) and verified the specificity using monoclonal antibody P2NA that exclusively detects human ErbB-2 phosphorylated at tyrosine residue 1248 (not shown).

In males and females, human ErbB-2 protein distribution in the cerebellum was prominent in the Bergman glia cells, along their fibrous extensions and on the membranes of the purkinje cells which interface the molecular layer and the granular layer where the transgene is not expressed. This localization may indicate an association with the endogenous ErbB-3, which is expressed in the Bergman glia fibers and purkinje cells in adult cerebellum (22,23). Other WAP-transgenes have been expressed in the brain, under hormonal regulation (24) or present in other regions of the brain (17). Constitutive ErbB-2 expression in the cerebellum appears unique to our transgenic strain.

Compared to other organs tested, (including salivary gland, kidney, thymus, esophagus, adrenals, ovaries and testes), ErbB-2 expression in the brain has been most consistent and at the highest level in Her-2 Tg mice from F1 to B12 generation independent of sex, lactational status and parity (not shown). None of the tissues expressing human ErbB-2 demonstrated obvious abnormality. The mice were healthy and have normal life span.

Immune tolerance to ErbB-2

To determine if Her-2 Tg mice were tolerant to ErbB-2, growth of an immunogenic ErbB-2 bearing tumor was tested (Figure 4). In normal C57BL/6 mice, injection of 5 x 10^6

ID8 ovarian cancer cells resulted in tumor growth after approximately two months (19). Transfection of ID8 with ErbB-2 (ID8/E2) increased the immunogenicity of the tumor and C57BL/6 mice rejected ID8/E2 tumor cells. Her-2 Tg mice injected with 5 x 10^6 ID8/E2 cells began developing tumors after 5 months and all mice were tumor positive by 9 months (Figure 4). Transgene negative littermates did not develop ID8/E2 tumors ***($p<0.01$ when compared to Her-2 Tg mice)***. Flow cytometric analysis of ascites tumor from Her-2 Tg mice showed ErbB-2 expression on the majority of cells. Therefore, there was not a selection for ErbB-2 negative ID8 cells in Her-2 Tg mice.

To examine the induction of anti-ErbB-2 antibodies in Her-2 Tg mice, serum was collected three months after ID8/E2 tumor cell injection. Anti-ErbB-2 antibodies were not detected in Her-2 Tg mice while a significant induction of anti-ErbB-2 IgG antibodies in transgene negative littermates was detected ***($p<0.05$)*** (Figure 5). Antibodies against the parental ID8 tumor were not detected in either group (Figure 5). These results indicated that Her-2 Tg mice were tolerant to ErbB-2 and this tolerance was not broken by the growth of an ErbB-2 overexpressing tumor.

Anti-Tumor Immunity Induced in Her-2 Tg mice by DNA Vaccination

In a pilot study, we tested the induction of humoral immunity with pCMV-ErbB-2. After four i.m. injections with 100 μg DNA, 2 weeks apart, anti-ErbB-2 IgG was detected in 2/10 Her-2 Tg mice, whereas 3/3 transgene negative littermates generated high levels of anti-ErbB-2 specific antibodies (not shown). To overcome tolerance in Her-2 Tg mice, we subjected Her-2 Tg mice and their transgene negative littermates to a more robust DNA vaccination regimen. Her-2 Tg mice were co-vaccinated four times with pCMV secE2 encoding a secreted ErbB-2 extracellular domain and DNA encoding GM-CSF. Sera was collected one week after the fourth DNA vaccination and anti-ErbB-2 IgG antibodies were

measured ***as an indicator of anti-ErbB-2 immune response***. Transgene negative mice had significant anti-ErbB-2 IgG in their serum with an average of 32 ± 14 μg/ml (Figure 6). Low level anti-ErbB-2 antibodies were detected in 8 of 9 Her-2 Tg mice. One Her-2 Tg mouse had 43 μg/ml of anti-ErbB-2 IgG indicating that tolerance to ErbB-2 was clearly overcome in this mouse. Anti-ErbB-2 antibodies were not detected in any unvaccinated Her-2 Tg mice.

To test the effect of vaccination on tumor growth, vaccinated mice were boosted once more and challenged 2 wks later with EL-4/E2 cells. All transgene negative littermates were protected against EL-4/E2 challenge (Figure 7). In contrast, only 33% of Her-2 Tg mice were protected. ***Using log rank test, the protection of vaccinated Her-2 Tg mice was less than that of vaccinated transgene negative littermates ($p < 0.05$), but greater than that of control vector injected Her-2 Tg mice ($p < 0.05$). Therefore, five courses of i.m. injection with DNA encoding human ErbB-2 partially overcame humoral and possibly cellular tolerance to protect mice from tumor growth***

Discussion

A human ErbB-2 transgenic mouse strain (Her-2 Tg) syngeneic to C57Bl/6 background has been established. Strong expression of human ErbB-2 was detected in lactationally active mammary epithelium and a high level of expression was detected constitutively in the brain of both male and female mice. Several other organs demonstrated variable expression. Her-2 Tg mice were tolerant to human ErbB-2 and permissive to the out-growth of tumors expressing human ErbB-2 without generating an antibody response. DNA vaccination in Her-2 Tg mice produced a modest but detectable anti-ErbB-2 antibody response ***and 33% of the animals were protected from tumor growth*** although 100% of the transgene negative mice were protected. ***Induction of anti-ErbB-2 antibody was a sensitive***

indicator of immune response to ErbB-2 in the otherwise tolerant hosts, but cellular immunity may contribute significantly to tumor rejection as antibody level did not correlate directly with tumor rejection.

These results are comparable to those found in neu transgenic mouse models. Transgenic mice expressing either normal or activated rat neu demonstrated tolerance to neu antigen. In FVB Neu-N transgenic mice expressing MMTV-neu, immunization with irradiated whole cell or recombinant vaccinia virus induced very weak cellular and humoral response when compared to FVB mice (25). In BALB NeuT mice expressing MMTV-NeuT, neu specific antibody and anti-tumor immunity was induced by vaccination with Neu DNA, although the level of response is much reduced when compared with transgene negative littermates (26). Our findings demonstrate that tolerance in Her-2 Tg mice was partially overcome by DNA vaccination. This model enables us to test human ErbB-2 based vaccination strategies in a realistic, tolerant host.

Previous report demonstrated that MMTV-Her-2 transgenic mice died within four months of age, probably due to kidney and lung failure following the development of preneoplastic lesion (16). A viable Sprague-Dawley rat transgenic for the wildtype human ErbB-2 gene has been described (27). In this model, expression of the transgenic mRNA (under the control of the MMTV-LTR) was detectable in mid-pregnant but not virgin mammary tissues. After repeated cycles of pregnancy and lactation, pathological changes were produced in the mammary glands.

Using WAP promoter to express c-ErbB-2, Her-2 Tg mice developed normally without detectable lesions in the lung or kidney. ErbB-2 expression in the mammary gland was significant. The consistent and high level expression in the brain has been reported before with WAP promoter regulated transgenes. Human growth hormone driven by WAP promoter was highly expressed in the brain of both male and female transgenic mice (21).

Mice carrying WAP promoter regulated human urokinase-type plasminogen activator also demonstrated consistent transgene expression in the brain extract (28). Expression of human ErbB-2 in the brain did not have detectable pathological consequence in Her-2 Tg mice.

In normal human brain, ErbB-2 was detected consistently in oligodendrocytes, astrocytes and microglial cells and the level was elevated in patients with multiple sclerosis (29). ErbB-2 expressed in hypothalamic astrocytes mediates neuroendocrine functions. Luteinizing hormone-releasing hormone (LHRH) is released following stimulation of ErbB-2/4 complex on astrocytes by neuregulin, resulting in the development of female reproductive capacity (30). ErbB-2 is expressed in 86% of medulloblastoma, although it is not detected at any stage of cerebellar development, suggesting de-regulation of ErbB-2 during medulloblastoma tumorigenesis (22). Cranial Ganglia defects were detected in ErbB-2 knock-out mice (31). ErbB-2 appears to play critical roles in normal and diseased brain. Further analysis of ErbB-2 in Her-2 Tg mouse brain may provide useful information. ***Vaccination of Her-2 Tg mice did not result in detectable autoimmune symptoms. Mice lived and reproduced normally. When Her-2 DNA vaccination efficacy is enhanced by increasing DNA uptake or modulating regulatory T cell, autoimmunity may be manifested and this should be closely monitored.***

FVB NeuN mice crossed with C57Bl/6 mice exhibit attenuated mammary tumor formation and increased tumor latency (32) and female mice develop tumors only after they experienced 2-4 pregnancies (33). The influence of strain background on mammary gland lesion incidence and phenotypes in WAP-TGFα transgenics has recently been described by Rose-Hellekant et al (34). Here, we show that expression of WAP-ErbB-2 in C57Bl/6 background resulted in a Her-2 tolerant mouse strain without spontaneous tumor growth. This strain will be valuable for testing ErbB-2 based vaccination against different tumor types. They can also be bred with mice expressing human HLA transgene to test human ErbB-2

vaccination in different HLA background. If spontaneous tumors are desired, Her-2 Tg mice can be bred with various oncogene transgenic or tumor suppressor gene knockout mice to produce tumors of different nature.

Acknowledgement

The authors wish to thank Dr. Bernd Groner for his advice and support and ***Mr. David Shim and Kevin Tse for their excellent technical assistance***.

Figure Legends

Figure 1. Construction of Her-2 transgene and genotypic identification of Her-2 Tg mice. (A) A schematic representation of WAP regulated Human ErbB-2 (Her-2) transgene. The transgene is a 6939 bp linear HinDIII fragment consisting of the 2.6 kB mouse whey acidic protein promoter fused with downstream 4.4 kB human ErbB-2 cDNA. Polyadenylation sequence at the 3' end of the gene are from the bovine growth hormone gene. The shaded arrows indicated the location of PCR primers used in genotyping. Probe for Southern blotting and the relevant EcoRI restriction sites are indicated. (B) Expression of the human ErbB-2 transgene. D2F2 mouse mammary tumor cells were co-transfected with the WAP-Her-2 cassette and pRSV-neo. Stable clones expressing human ErbB-2 were selected and cultured in the absence (left panel) or presence (right panel) of 10 μM hydrocortisone and 10 μg/mL insulin for 48 hours. Cell surface expression of human ErbB-2 was evaluated by flow cytometry with monoclonal antibody TA-1 and detected with FITC conjugated goat anti-mouse secondary antibody (shaded histogram). The clear histogram represents binding of an isotype control antibody. (C) Genotype analysis of Her-2 Tg mice. Positive identification of transgenic animals is indicated by hybridization of the probe to a 1.6 kB EcoRI fragment (Top) and by the amplification of a 352 bp PCR product (Bottom). Southern blot and PCR analyses were performed on genomic DNA from the same ten pups and the results demonstrated perfect concordance.

Figure 2. Expression of ErbB-2 protein in Her-2 Tg mice. Immunoprecipitation and Western blot analysis of human ErbB-2 gene product in the mammary tissue of F1 females at one to three days post-partum. Mammary tissues were removed aseptically and placed in ice-cold lysis buffer. Tissues were minced and proteins immunoprecipitated with the anti-ErbB-2 humanized monoclonal antibody, Herceptin. Proteins were resolved in SDS-PAGE,

transferred to Immobilon-P and hybridized with mAb 3B5 specific for a carboxy terminal epitope of ErbB-2. Bound antibodies were detected with HRPO conjugated goat anti-mouse Ig and visualized with chemiluminescent substrate. Lane 1 is a positive control of D2F2/E2 cells transfected with human ErbB-2. The arrow points to the 185 kD human ErbB-2. Lane 2 is the molecular weight standards. Lanes 4-7 are proteins immunoprecipitated from mammary tissues of lactating transgene positive females. Lanes 4 + 5 represent two F1 founders and lanes 6 + 7 are the offspring of the F1 in lane 5. Lane 8 shows proteins from the mammary tissues of a non-transgenic lactating female. Lane 3 is whole tissue lysate from the F1 in lane 5.

Figure 3. Expression of ErbB-2 protein in Her-2 Tg mice fully-backcrossed (11 generations) onto the C57Bl/6 background. (A) Immunoprecipitation and Western blot analysis of human ErbB-2 gene product in the mammary tissue and cerebellum at lactation day (17-19). Tissues were minced and proteins were immunoprecipitated with monoclonal antibodies specific for different epitopes of the human Erb-B2 protein: 9G6.10, (lanes 2 and 4) or Herceptin (lanes 3 and 5) in mammary (lanes 2 and 3) or cerebellum (lanes 4 and 5) tissue lysates. Proteins were detected using the polyclonal antibody C-18 (Santa Cruz), Lane 1 is a positive control cerebellum tissue lysate. The arrow points to the 185 kD human ErbB-2. B) Immunohistochemical localization of human ErbB-2 in the mammary gland (a, b) and cerebellum (c, d) of a lactating (day 17-19) Her2-Tg female from the 11th backcross onto the C57Bl/6 background. Secretory mammary epithelia lining mature ductules and cross-sections of terminal endbud clusters exhibit intensely positive membrane staining (arrows) while supporting stromal cells and fibroblasts are clearly negative (asterisks). These mature lobulo-alveolar structures are characterized by single layers of secretory epithelial cells with few adipocytes. In addition, the non-secretory immature, undifferentiated mammary gland

cells failed to demonstrate robust membrane ErbB-2 staining. Expression of human ErbB-2 in the cerebellum of Her-2 Tg female (c, d) and male (e, f) mice. Intense immunoreactivity using the TAB250 human-ErbB-2 specific monoclonal antibody was observed in the molecular layer (ML) of the cerebellum (Figure d + f). Distribution of the protein was prominent along the Bergman glial fibers (arrows) and to a lesser extent in the membranes of the purkinje cells (PCL), but not evident in the granular layer (GL). (C) Immuno-precipitation and Western blot analysis of human ErbB-2 gene product in salivary gland and brain tissues of male Her-2 Tg mice. Human ErbB-2 protein is exclusively expressed in the cerebellum of males from the 10th backcross (lane 3). No human ErbB-2 protein was detected in the cerebrum (lane 4) parotid (lane 1), other salivary glands (lane 2) nor in any of these corresponding tissues in transgene negative littermates (lanes 5-8). (D) The immunoprecipitated human ErbB-2 in the cerebellum is phosphorylated on tyrosine residues as detected by the anti-phosphotyrosine monoclonal antibody, PY20-HRPO.

Figure 4. Her-2 Tg mice developed immunogenic ID8/E2 tumors. Her-2 Tg + (n=4) and Her-2 Tg- mice (n=4) from the 8th generation backcross, were injected i.p. with 5x10^6 ID8/E2 cells in 0.5 ml PBS. Mice were palpated weekly for the onset of i.p. ascites. Mice were sacrificed when i.p. ascites were apparent. The arrow above the 3 month interval denotes the time at which sera was sampled to determine levels of anti-ErbB-2 and anti-ID8 specific antibodies (graphed in Figure 5). ***Statistical significance was determined by the log-rank test.***

Figure 5. Anti-ErbB-2 antibodies were not induced in Her-2 Tg mice. Her-2 Tg mice (Her-2 Tg +) and non-transgenic littermates (Her-2 Tg-) (n=4) were injected i.p. with 5 x 10^6 ID8

cells transfected with ErbB-2. Three months later, serum was collected and used to stain SKBR-3 (A) or ID8 (B) cells. Bound antibody was detected by flow cytometry. The results are expressed as the mean channel fluorescence (MCF) of individual samples. * indicates $p<0.05$ by the Student's t test as compared to antibody production in Her-2 Tg mice.

Figure 6. Induction of anti-ErbB-2 antibodies in Her-2 Tg mice. Her-2 Tg mice (n=9) and negative littermates (n=12) from the 8th generation backcross, were immunized four times with 100 μg each of pSecE2 and GM-CSF DNA. Control Her-2 Tg mice (n=4) were not vaccinated. Sera were collected after the final DNA vaccination and anti-ErbB-2 IgG antibody was measured by its binding to SKBR-3 cells and measured by flow cytometry. The results are expressed as μg/ml of individual samples. Asterisk (*) indicates $p < 0.001$ by the Student's t test as compared to Her-2 Tg mice.

Figure 7: Anti-tumor immunity induced in Her-2 Tg mice. Her-2 Tg mice (▲) and non-transgenic littermates (◇) from the 8th generation backcross, were immunized i.m. five times with 100 μg each of pSec-E2 + GM-CSF. At two weeks after the last immunization, mice were challenged s.c. with $2x10^5$ EL-4/E2 cells and included a group (n=4) Her-2 Tg mice that were not immunized to serve as positive controls for tumor growth (■). Tumor growth was measured by weekly palpation. ***Statistical significance was determined by the log-rank test.***

References

1. Slamon, D.J., W. Godolphin, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Levin, S.G. Stuart, J. Udove, A. Ullrich, and M.F. Press. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science 244:707.*

2. Yu, D. and M.C. Hung. 2000. Overexpression of ErbB2 in cancer and ErbB2-targeting strategies. *Oncogene 19:6115.*

3. Tzahar, E. and Y. Yarden. 1998. The ErbB-2/HER2 oncogenic receptor of adenocarcinomas: from orphanhood to multiple stromal ligands. *Biochem. Biophys. Acta 1377:M25.*

4. Disis, M., E. Calenoff, G. McLaughlin, A.E. Murphy, W. Chen, B. Groner, M. Jeschke, N. Lydon, E. McGlynn, R.B. Livingston, R. Moe, and M.A. Cheever. 1994. Existent T cell and antibody immunity to Her-2/neu protein in patients with breast cancer. *Cancer Res. 54:16.*

5. Peoples, G.E., P.S. Goedegebuure, R. Smith, D.C. Linehan, I. Yoshino, and T.J. Eberlein. 1995. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same Her-2/neu-derived peptide. *Proc. Nat. Acad. Science 92:432.*

6. Fisk, B., B.W. Anderson, K.R. Gravitt, C.A. O'Brian, A.P. Kudelka, J.L. Murray, J.T. Wharton, and C.G. Ioannides. 1997. Identification of naturally processed human ovarian peptides recognized by tumor-associated cytotoxic T lymphocytes. *Cancer Res. 57:87.*

7. Kobayashi, H., M. Wood, Y. Song, E. Appella, and E. Celis. 2000. Defining promiscuous MHC class II helper T-cell epitopes for the HER2/*neu* tumor antigen. *Cancer Res. 60:5228.*

8. Cobleigh, M.A., C.L. Vogel, D. Tripathy, N.J. Robert, S. Scholl, L. Fehrnbacher, J.M. Wolter, V. Paton, S. Shak, G. Lieberman, and D.J. Slamon. 1999. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J.Clin.Oncol. 17:2639.*

9. Wei, W.Z., W.P. Shi, A. Galy, D. Lichlyter, S. Hernandez, B. Groner, L. Heilbrun, and R.F. Jones. 1999. Protection Against Mammary Tumor Growth By Vaccination With Full-Length, Modified Human *ErbB-2* DNA. *Int. J. Cancer 81:748.*

10. Piechocki, M.P., S. Pilon, and W.-Z. Wei. 2001. Complementary antitumor immunity induced by plasmid DNA encoding secreted and cytoplasmic human ErbB-2. *J. Immunol. 167:3367.*

11. Pilon, S., M.P. Piechocki, and W.-Z. Wei. 2001. Vaccination with cytoplasmic ErbB-2 DNA protects mice from mammary tumor growth without anti-ErbB-2 antibody. *J. Immunol. 167:3201.*

12. Guy, C.T., M.A. Webster, M. Schaller, T.J. Parsons, R.D. Cardiff, and W.J. Muller. 1992. Expression of the neu protooncogene in the mammry epithelium of transgenic mice induces metastatic disease. *Proc. Nat. Acad. Science 89:10578.*

13. Boggio, K., G. Nicoletti, E. Di Carlo, F. Cavallo, L. Landuzzi, C. Melani, M. Giovarelli, I. Rossi, P. Nanni, C. De Giovanni, P. Bouchard, S. Wolf, A. Modesti, P. Musiani, P.L. Lollini, M.P. Colombo, and G. Forni. 1998. Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two cell-lines of Her-2/*neu* transgenic mice. *J. Exp. Med. 188:589.*

14. Yamamoto, T., S. Ikawa, T. Akiyama, K. Semba, N. Nomura, N. Miyajima, T. Saito, and K. Tohoshima. 1986. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. *Nature 319:230.*

15. ***Pupa, SM., AM. Invernizzi, S. Forti, E. Di Carlo, P. Musiani, P. Nanni, PL. Lollini, R. Meazza, S. Ferrini, and S. Menard. 2001. Prevention of spontaneous neu-expressing mammary tumor development in mice transgenic for rat proto-neu by DNA vaccination. Gene Therapy 8: 75.***

16. Stocklin, E., F. Botteri, and B. Groner. 1993. An activated allele of the c-*erbB-2* oncogene impairs kidney and lung function and causes early death of transgenic mice. *The Journal of Cell Biology 122:199.*

17. Wen J., Y. Kawamata, H. Tojo, S. Tanaka and C. Tachi. 1995. Expression of whey acidic protein (WAP) genes in tissues other than the mammary gland in normal and transgenic mice expressing mWAP/hGH fusion gene. *Mol. Rep. and Dev. 41, 399.*

18. Mahoney, K.H., B.E. Miller, and G.H. Heppner. 1985. FACS quantitation of leucine aminopeptidase and acid phosphatase on tumor associated macrophages from metastatic and nonmetastatic mouse mammary tumors. *J. Leuk. Biol. 38:573.*

19. Roby KF, Taylor CC, Sweetwood JP, Cheng Y, Pace JL, Tawfik O, Persons DL, Smith PG, Terranova PF. 2000. Development of a syngeneic mouse model for events related to ovarian cancer. *Carcinogenesis. 21(4):585.*

20. Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994. *Manipulating the mouse embryo: A laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

21. Gunzburg, W.H., B. Salmons, B. Zimmermann, M. Muller, V. Erfle, and G. Brem. 1991. A mammary-specific promoter directs expression of growth hormone not only to the mammary gland, but also to bergman glia cells in transgenic mice. *Mol. Endocrinol. 5:123.*

22. Gilbertson R. J., S. C. Clifford, W. MacMeekin, W. Meekin , C. Wright , R. H. Perry, P. Kelly, A. D. Pearson and J. Lunec . 2001. Expression of the ErbB-neuregulin signaling network during human cerebellar development: implications for the biology of medulloblastoma. *Cancer Res. 58, 3932.*

23. Pinkas-Kramarski R, Eilam R, Alroy I, Levkowitz G, Lonai P, Yarden Y. 1997. Differential expression of NDF/neuregulin receptors ErbB-3 and ErbB-4 and involvement in inhibition of neuronal differentiation. *Oncogene. 15(23):2803.*

24. Andres AC, Schonenberger CA, Groner B, Hennighausen L, LeMeur M, Gerlinger P. 1987. Ha-ras oncogene expression directed by a milk protein gene promoter: tissue

specificity, hormonal regulation, and tumor induction in transgenic mice. *Proc. Nat. Acad. Science 84(5):1299.*

25. Reilly, R.T., M.B.C. Gottlieb, A.M. Ercolini, J.P.H. Machiels, C.E. Kane, F.I. Okoye, W.J. Muller, K.H. Dixon, and E.M. Jaffee. 2000. HER-2/*neu* is a tumor rejection target in tolerized HER-2/*neu* transgenic mice. *Cancer Res. 60*:3569.

26. Rovero, S., A. Amici, E. Di Carlo, R. Bei, P. Nanni, E. Quaglino, P. Porcedda, K. Boggio, A. Smorlesi, P.L. Lollini, L. Landuzzi, M.P. Colombo, M. Giovarelli, P. Musiani, and G. Forni. 2000. Inhibition of carcinogenesis by DNA vaccination. *J. Immunol. 165*:5133.

27. Davies BR, Platt-Higgins AM, Schmidt G, Rudland PS. 1999. Development of hyperplasias, preneoplasias, and mammary tumors in MMTV-c-erbB-2 and MMTV-TGFalpha transgenic rats. *Am. J. Pathol. 155(1):303.*

28. Brandazza A, E. Lee, M. Ferrera, U. Tillman, P. Sarmientos and H. Westphal. 2001. Use of the urokinase-type plasminogen activator gene as a general tool to monitor expression in transgenic animals: study of the tissue-specificity of the murine whey acidic protein (WAP) expression signals. *J Biotechnol. 20:201.*

29. Cannella, B., D. Pitt, M. Marchionni, and C.S. Raine. 1999. Neuregulin and erbB receptor expression in normal and diseased human white matter. *J. Neuroimmunol. 100*:233.

30. Ma Y. J., D. F. Hill, K. E. Creswick, M. E. Costa, A. Cornea, M. N. Lioubin, G. D. Plowman and S. R. Ojeda. 1999. Neuregulins signaling via a glial erbB-2-erbB-4 receptor complex contribute to the neuroendocrine control of mammalian sexual development. *J. Neuroscience 19: 9913.*

31. Erickson S. L., K. S. O'Shea , N. Ghaboosi , L. Loverro, G. Frantz, M. Bauer, L. H. Lu, and M. W. Moore. 1997. ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development 124:4999.*

32. Rowse GJ, Ritland SR, Gendler SJ. 1998. Genetic modulation of neu proto-oncogene-induced mammary tumorigenesis. *Cancer Res. 58(12):2675.*

33. Liu S, Liu W, Jakubczak JL, Erexson GL, Tindall KR, Chan R, Muller WJ, Adhya S, Garges S, Merlino G. 2002. Genetic instability favoring transversions associated with ErbB2-induced mammary tumorigenesis. *Proc. Nat. Acad. Science 99(6):3770.*

34. Rose-Hellekant TA, Gilchrist K, Sandgren EP. 2002. Strain background alters mammary gland lesion phenotype in transforming growth factor-alpha transgenic mice. *American J. Pathol. 161(4):1439.*

Figure 1

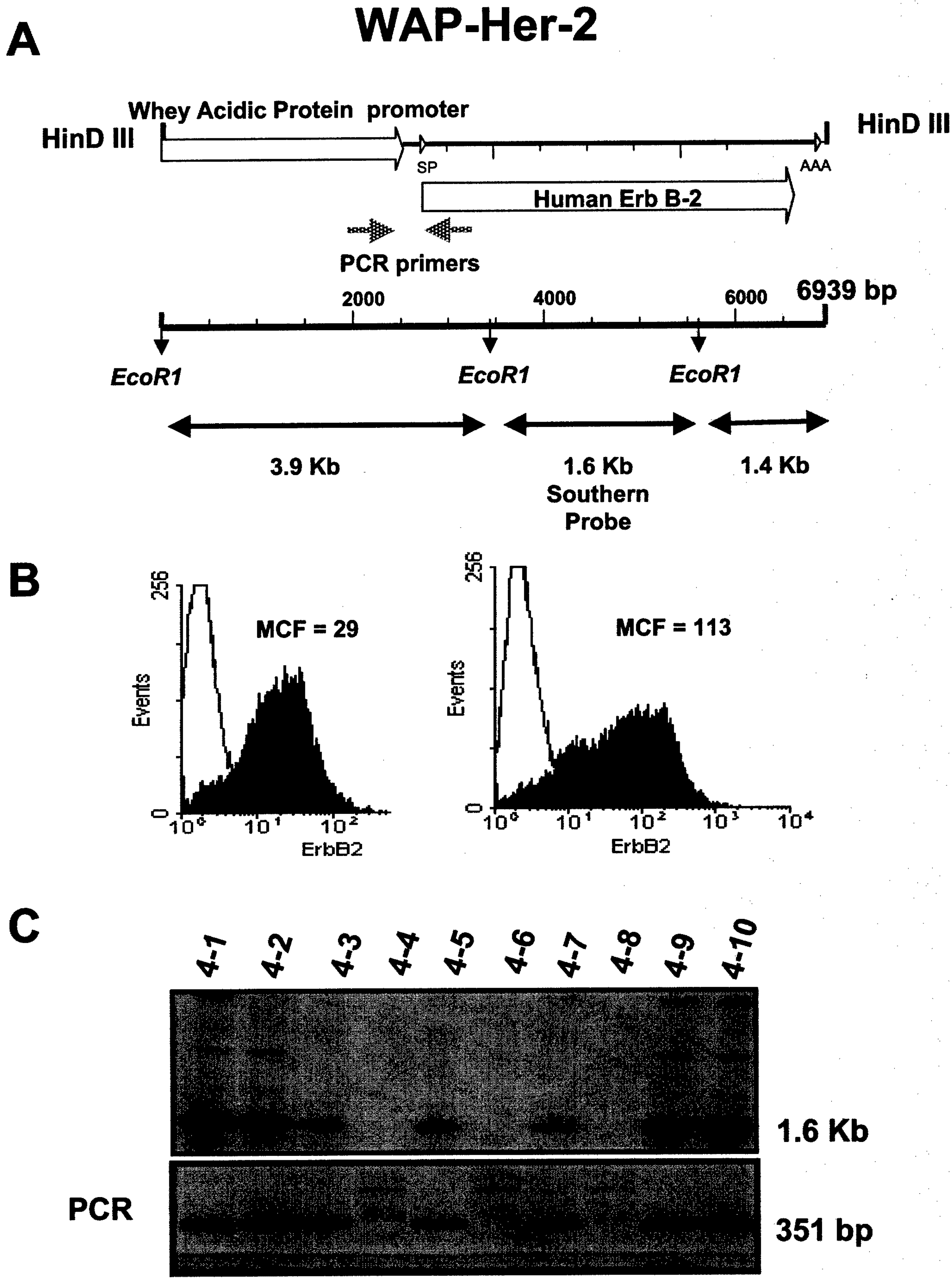
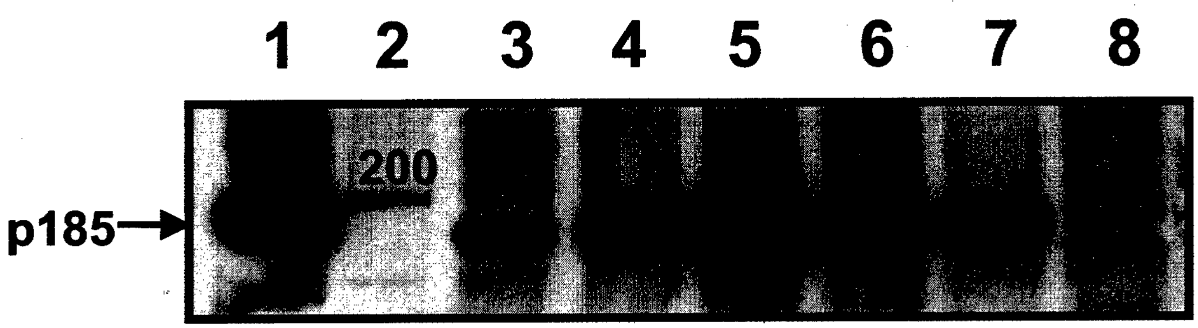
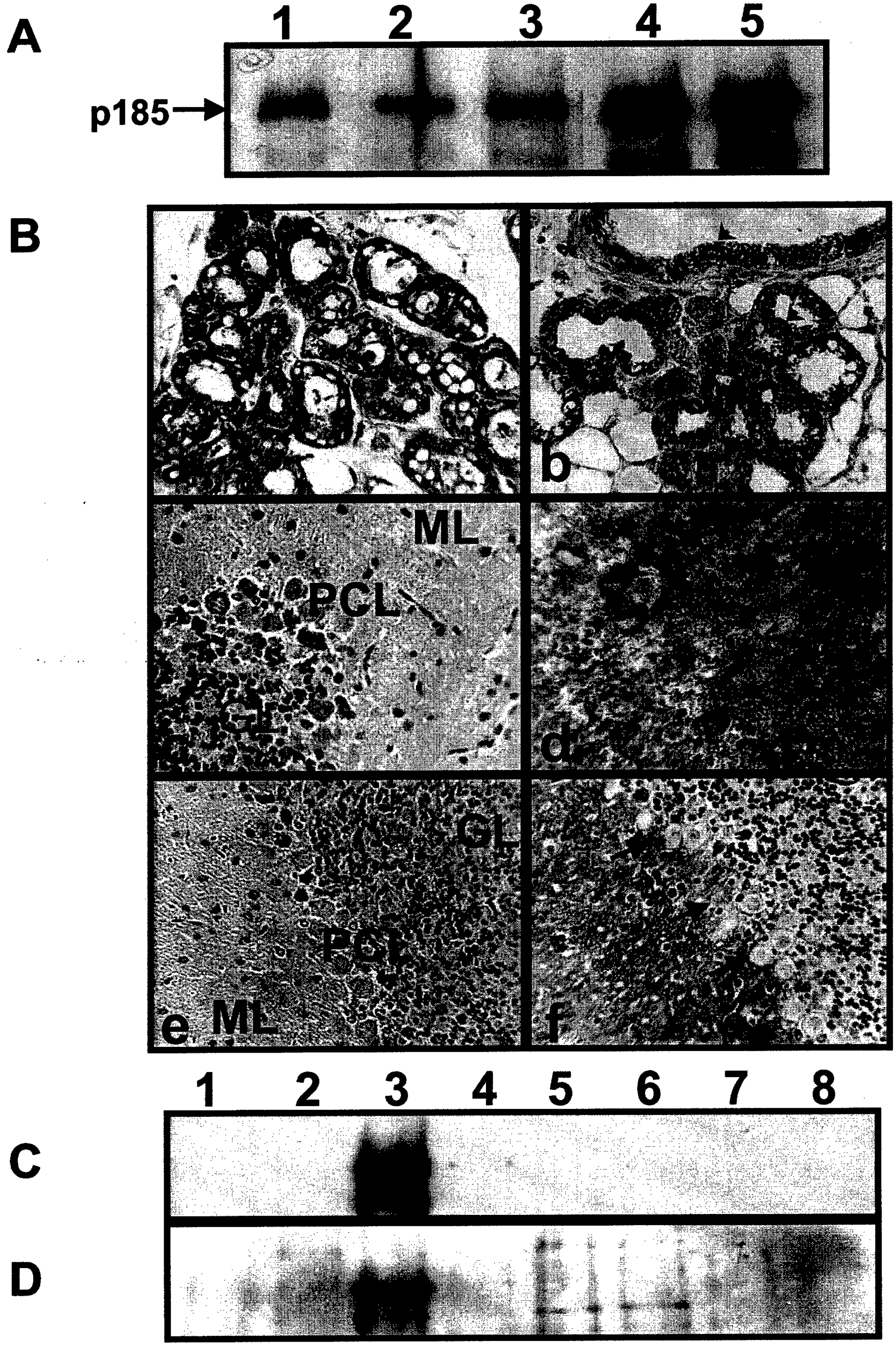

A
WAP-Her-2
Whey Acidic Protein promoter
HinD III
HinD III
SP
AAA
Human Erb B-2
PCR primers
2000
4000
6000
6939 bp
EcoR1
EcoR1
EcoR1
3.9 Kb
1.6 Kb
Southern
Probe
1.4 Kb
B
256
0
Events
MCF = 29
10^0
10^1
10^2
ErbB2
256
0
Events
MCF = 113
10^0
10^1
10^2
10^3
10^4
ErbB2
C
4-1
4-2
4-3
4-4
4-5
4-6
4-7
4-8
4-9
4-10
1.6 Kb
PCR
351 bp

Figure 2





A
1
2
3
4
5
p185
B
a
b
ML
PCL
GL
d
GL
PCL
e
ML
f
1
2
3
4
5
6
7
8
C
D

Figure 4

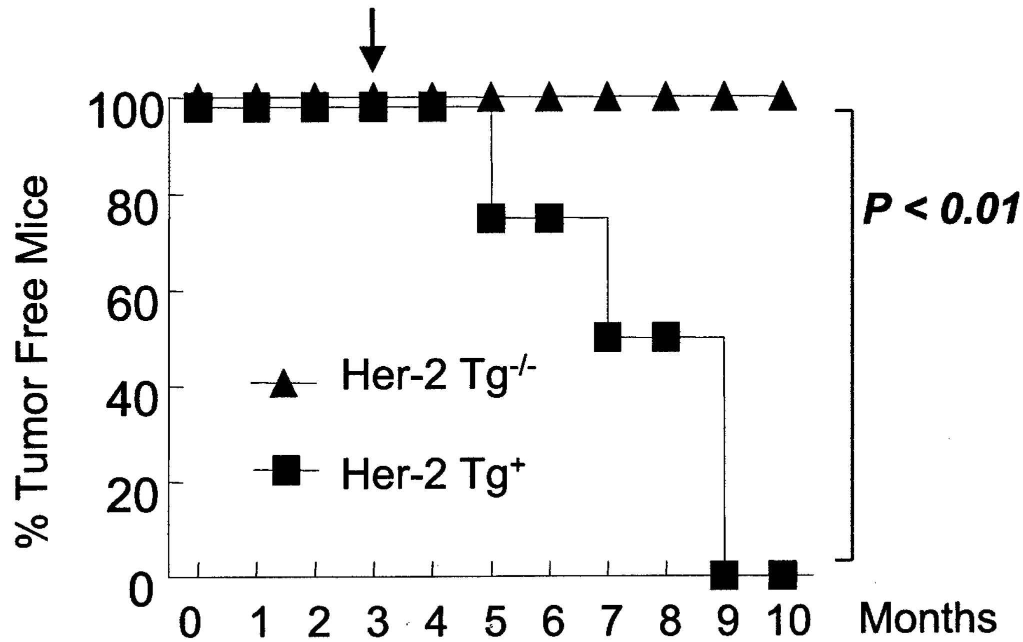


Figure 5

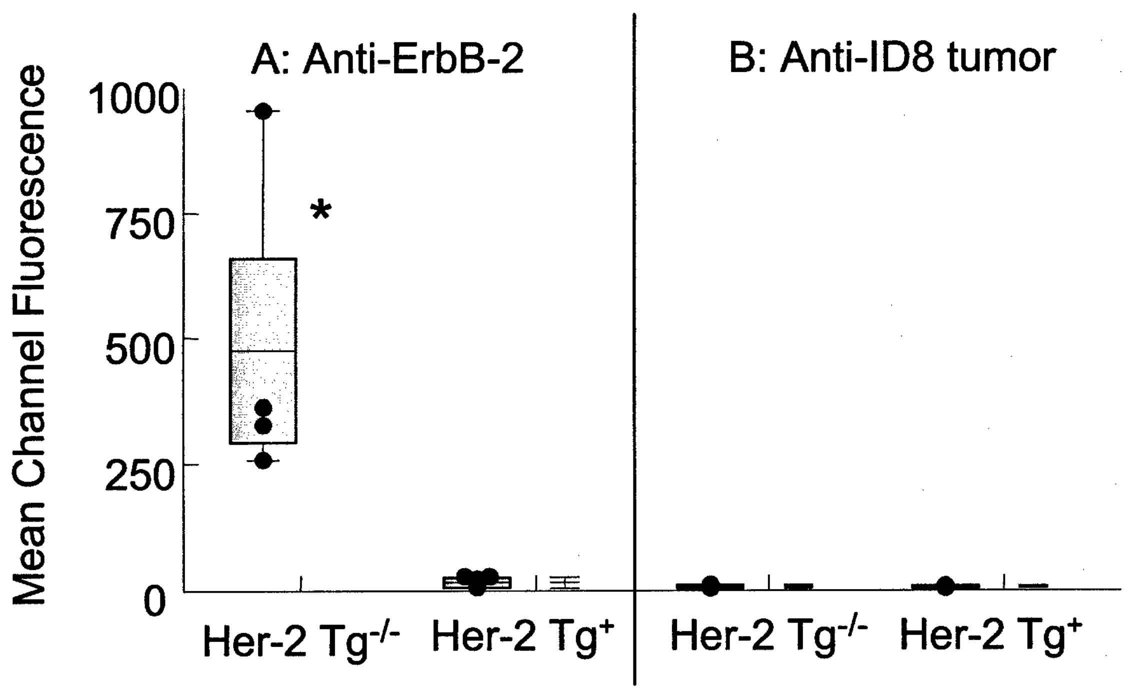


Figure 6

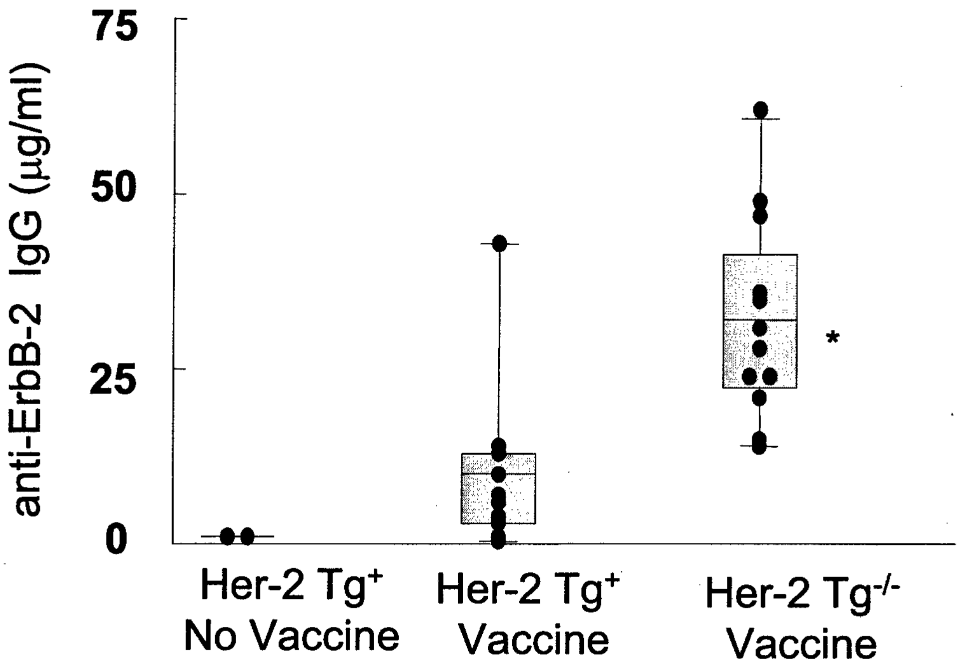


Figure 7